TITLE: **HEME PROTEINS HemAT-*Hs* AND HemAT-*Bs* AND THEIR USE IN MEDICINE AND MICROSENSORS**

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DOCKET: **201040/1020**

HEME PROTEINS HEMAT-*HS* AND HEMAT-*BS* AND THEIR USE IN MEDICINE AND MICROSENSORS

The subject matter of this application was made with support from the United States Government under Grant No. MSB960086 from the National Science Foundation. The United States Government may retain certain rights.

BACKGROUND OF THE INVENTION

Heme proteins such as hemoglobin and myoglobin play an essential role in stabilizing molecular oxygen for transport and storage. The oxygen carrying portion of the red blood cell is hemoglobin, a tetrameric protein molecule composed of two identical alpha globins (alpha 1, alpha 2), two identical beta globins (beta 1, beta 2) and four heme molecules. A heme molecule is incorporated into each of the alpha and beta globins to give alpha and beta subunits. Heme is a macrocyclic organic molecule that contains an iron atom at its center; each heme can combine reversibly with one ligand molecule, for example oxygen. In a hemoglobin tetramer, each alpha subunit is associated with a beta subunit to form two stable alpha/beta dimers, which in turn associate to form the tetramer (a homodimer). The subunits are noncovalently associated through Van der Waals forces, hydrogen bonds and salt bridges. Ligands, particularly oxygen, bind reversibly to the reduced form of the iron (ferrous, Fe^{2+}) in the heme. Other ligands which compete with oxygen for the heme group include carbon monoxide and nitric oxide.

It is not always practical to transfuse a patient with donated blood. The well known complications of blood transfusion namely incompatibility reactions, disease transmission, immunosuppression and the storage limitations of erythrocytes points to the need for the development of blood substitutes devoid of these shortcomings. In these situations, use of a red blood cell substitute is necessary. A "blood substitute" is a preparation that does not necessarily replace blood in all of its functions, but an emergency resuscitative fluid that is capable of efficiently transporting oxygen to tissue. This fluid, however, must be free of toxic side-effects, as well as of agents of disease such as bacteria and viruses.

For over 50 years, efforts directed to the development of a blood substitute have focused on hemoglobin (Hb). Hemoglobin (Hgb) is the oxygen-carrying component of

blood. Hemoglobin circulates through the bloodstream inside small enucleate cells called erythrocytes (red blood cells). Hemoglobin is a protein constructed from four associated polypeptide chains, and bearing prosthetic groups known as hemes. The erythrocyte helps maintain hemoglobin in its reduced, functional form. The heme iron atom is labile to oxidation, but may be reduced again by one of two enzyme systems within the erythrocyte, the cytochrome b5 and glutathione reduction systems.

Hemoglobin exhibits cooperative binding of oxygen by the four subunits of the hemoglobin molecule (two alpha-globins and two beta-globins in the case of HbA), and this cooperativity greatly facilitates efficient oxygen transport. Cooperativity, achieved by the so-called heme-heme interaction, allows hemoglobin to vary its affinity for oxygen. Hemoglobin reversibly binds up to four moles of oxygen per mole of Hb. At high oxygen concentration, such as that found in the lungs, the oxygen affinity is high and hemoglobin is almost saturated with oxygen. At low oxygen concentration, such as that found in actively respiring tissue, the oxygen affinity is lowered and oxygen is unloaded. The oxygen affinity of hemoglobin is lowered by the presence of 2,3-diphosphoglycerate (2,3-DPG), chloride ions and hydrogen ions. Respiring tissue releases carbon dioxide into the blood and lowers its pH (i.e. increases the hydrogen ion concentration), thereby causing oxygen to dissociate from hemoglobin and allowing it to diffuse into individual cells.

The ability of hemoglobin to alter its oxygen affinity, increasing the efficiency of oxygen transport around the body, is dependent on the presence of the metabolite 2,3-DPG. Inside the erythrocyte 2,3-DPG is present at a concentration nearly as great as that of hemoglobin itself. In the absence of 2,3-DPG "conventional" hemoglobin binds oxygen very tightly and would release little oxygen to respiring tissue.

Aging erythrocytes release small amounts of free hemoglobin into the blood plasma where it is rapidly bound by the scavenging protein haptoglobin. The hemoglobin-haptoglobin complex is removed from the blood and degraded by the spleen and liver.

It is clear from the above considerations that free native hemoglobin A, injected directly into the bloodstream, would not support efficient oxygen transport about the body. The essential allosteric regulator 2,3-DPG is not present in sufficient concentration in the plasma to allow hemoglobin to release much oxygen at venous oxygen tension, and

free hemoglobin would be rapidly inactivated as an oxygen carrier by auto-oxidation of the heme iron.

Therefore, a need exists for a substitute other than hemoglobin which can bind and carry oxygen to cells. This substitute may also be used in other applications where hemoglobin is used, including as a biological sensor for oxygen. The present invention provides proteins which meet that need.

SUMMARY OF THE INVENTION

The present invention provides isolated archaeal and bacterial heme binding proteins which reversibly bind oxygen with a low affinity.

The invention also provides a blood substitute containing the bacterial heme binding protein which reversibly binds oxygen with a low affinity.

Another embodiment of the invention is a method for controlled storage of oxygen. A bacterial heme binding protein which reversibly binds oxygen with a low affinity is contacted with oxygen allowing the protein to bind and store oxygen. The invention also provides a method of sensing gaseous ligands. A heme binding bacterial protein is exposed to a test sample and a change in the conformation of the protein is measured.

Yet another embodiment of the invention is a chimeric protein having a heme-binding domain and a heterologous signaling domain.

The invention further provides an isolated nucleic acid molecule which encodes a heme binding bacterial protein that reversibly binds oxygen with a low affinity.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the conserved sequences within HemAT-*Hs*, HemAT-*Bs*, and sperm-whale myoglobin (SWMb). Black boxes indicate positions at which the residues are identical, and gray boxes highlight residues that are similar. Sequences were aligned using the Clustal program of the MegAlign/DNASTAR package. A) Alignment of the amino-terminal domain of HemAT-*Hs*, HemAT-*Bs*, and SWMb. Helical regions in SWMb (helices A-H) (B. C. Clothia, et al., J. Mol. Biol. 196:199 (1987); S. N. Vinogradov et al., Comp. Biochem. Physiol. 106B:1 (1993), which are hereby

incorporated by reference) are delineated by dotted arrows. Pro (P), Phe (F), and His (H) residues in SWMb that are highly conserved among all globins are marked with asterisks. B) Alignment of the carboxyl-terminal domains of HemAT-*Hs*, HemAT-*Bs*, and Tsr (B. K. Kendall, et al., Nature 301:623 (1983); G. L. Hazelbauer, Curr. Opin. Struct. Biol., 2:505 (1992), which are hereby incorporated by reference).

Figure 2 is a characterization of HemAT proteins. Figure 2A shows the purified HemAT-*Hs* and HemAT-*Bs* in 10% SDS-PAGE. Approximately 5 μg of purified protein were loaded in each lane for separation during SDS-PAGE in 10% acrylamide (M. Alam et al., J. Bacteriol., 173:5837 (1991), which is hereby incorporated by reference). Lane 1, HemAT-*Hs*; lane 2, $HemAT_{6xHis}$-*Hs*; and lane 3, HemAT-*Bs*. The MW markers (kDa) are shown at the left. Figure 2B is a fluorograph and immunoblot of HemAT-*Hs*. Radiolabeling and immunoblotting were performed as previously described (M. Alam et al., J. Bacteriol., 173:5837 (1991), which is hereby incorporated by reference). Lane 1, fluorograph of proteins from the Δ*hemAT-Hs*; lane 2, fluorograph of proteins from the Δ*hemAT-Hs*/*hemAT-Hs*++ strain (A. Brooun, Ph.D thesis. University of Hawaii, Hawaii (1997), which is hereby incorporated by reference). *Nde*I and *Xba*I restriction sites were used to clone the *hemAT-Hs* gene into the shuttle vector pKJ427. Primers introducing flanking *Nde*I and *Xba*I restriction sites were used for PCR amplification. The PCR product was initially cloned into the pCR*R*-Blunt II TOPO cloning vector and later subcloned into plasmid pKJ427 after digestion with *Nde*I and *Xba*I. The resulting plasmid was introduced into the Δ*hemAT-Hs* strain. Lane 1: immunoblot of Δ*hemAT-Hs* strain; and lane 2: immunoblot of Δ*hemAT-Hs*/*hemAT-Hs*++ strain using anti-transducer peptide antibody (W. Zhang et al., Proc. Natl. Acad. Sci. U.S.A. 93:4649 (1996), which is hereby incorporated by reference). Bars indicate the positions of molecular weight markers (kDa). Radiolabeling and immunoblot experiments were performed according to Alam & Hazelbauer (Alam et al., J. Bacteriol., 173:5837-5842 (1991)).

Figure 3 provides a comparison of the proteins used in the homology analyses. M1 and M2 are the site of myoglobin recognition. M2 is the site of HemAT recognition. The H-box is the primary site of microbial hemoglobin recognition.

Figure 4 shows absorption spectra of purified HemAT-*Hs*, HemAT-*Bs*, and horse-heart myoglobin (HHMb). Panel A shows oxygenated forms of purified HemAT-*Hs*, HemAT-*Bs*, and oxymyoglobin. Panel B shows deoxygenated forms of HemAT-*Hs*,

HemAT-*Bs*, and myoglobin. Panel C shows CO-bound forms of HemAT-*Hs*, HemAT-*Bs*, and myoglobin. Panel D shows reoxidized forms of HemAT-*Hs*, HemAT-*Bs*. Samples concentrations are approximately 20 μM in heme. Deoxygenated samples were prepared by the addition of sodium dithionite to the deaerated protein solutions.

Figure 5 shows aerotactic responses in *H. salinarum* and *B. subtilis*. Panel A shows *H. salinarum* strain Flx15 (HtrVIII and HemAT-*Hs* present), and mutant strains Δ*hemAT-Hs* (HtrVIII present) and ΔhtrVIII (HemAT-*Hs* present). Panel B shows wild-type *B. subtilis* strain OI1085 and mutant strains OI3545 (Δten) and OI3555 (overexpression of *hemAT-Bs* in Δten). All cells were grown to mid-logarithmic phase. Microcapillaries (internal dimension 100x10 μm) were filled halfway with cell suspension. The capillaries were sealed at both ends and placed on a microscope stage prewarmed to 35-37 °C. Time-lapse, dark-field microscopic images were recorded using a video-digitized camera linked to a computer. The images shown were taken at 180 min for *H. salinarum* and at 30 min for *B. subtilis*. The meniscus at the air interface is visible to the right in each image.

Figure 6 provides transient absorption data subsequent to CO photolysis obtained at 430 nm at 25°C and 1 atm CO for HemAT-*Hs* (solid line) and HemAT-*Bs* (dotted line). Samples were approximately 20 μM. The traces are the average of 50 laser pulses (532 nm exciation, 7 ns pulse width, 10 mJ/pulse).

Figure 7 shows the transient difference spectrum (25 μs subsequent to photolysis) overlaid with the equilibrium difference spectrum (deoxy minus CO-bound) for HemAT-*Hs* (top panel) and HemAT-*Bs* (bottom panel). Sample conditions are as described in Figure 4.

Figure 8 provides CO-off rate data for HemAT-*Hs* (dashed line), HemAT-*Bs* (dotted line), and horse heart Mb (solid line). Changes in absorbance as a function of time at 418 were monitored after the addition of potassium ferricyanide (final concentration of 1.5 mM). See Example 17 for details.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an isolated bacterial myoglobin-like heme binding protein which reversibly binds oxygen with a low affinity. These proteins are a new class of heme proteins that bind diatomic oxygen through their prosthetic group and trigger negative aerotactic responses. HemAT-*Hs* and HemAT-*Bs* are the first myoglobin-like heme proteins in the *Archaea* and in *Bacteria,* respectively. Purified HemAT-*Hs* and HemAT-*Bs* exhibit spectral properties similar to oxygen-bound myoglobin. Deoxygenation of either protein results in absorption shifts similar to those observed for deoxymyoglobin. The oxy-/deoxy spectral changes in HemAT-*Hs* and in HemAT-*Bs* are completely reversible, a characteristic feature of the heme prosthetic group in myoglobin. The C-terminus of both proteins has high homology with the signaling domain of bacterial methyl-accepting chemoreceptors and they mediate aerotaxis. By site-directed mutagenesis the fifth coordination site of the heme iron was identified in HemAT-*Hs* and HemAT-*Bs* comparable to myoglobin.

In a preferred embodiment of the invention, the isolated heme-binding protein has both a heme binding domain and a signaling domain.

When the *hemAT-Hs* gene was originally cloned, its product was predicted to be a soluble signal transducer (W. Zhang et al., Proc. Natl. Acad. Sci. U.S.A. 93:4649 (1996), which is hereby incorporated by reference). HemAT-*Bs* was identified in the *B. subtilis* genome-sequencing project as the product of an open-reading frame encoding a protein with marked similarities to methyl-accepting chemotaxis proteins (MCP) (F. Kunst et al., Nature 390:249 (1997), which is hereby incorporated by reference). The predicted translation products of the *hemAT-Hs* and *hemAT-Bs* genes, comprising 489 and 432 residues, respectively, exhibit two striking features: a) their amino-termini (residues 1-184 in HemAT-*Hs* and 1-175 in HemAT-*Bs*) display limited homology to myoglobin (Figure 1A); b) residues 222 to 489 of HemAT-*Hs* and 198 to 432 of HemAT-*Bs* are 30% identical to the cytoplasmic signaling domain of Tsr, an MCP from *Escherichia coli* (Figure 1B).

The residues absolutely conserved among all globins are the proximal His in the F helix (F8) and Phe in the CD region (CD1) (B. C. Clothia, et al., J. Mol. Biol. 196:199 (1987); S. N. Vinogradov et al., Comp. Biochem. Physiol. 106B:1 (1993), which are hereby incorporated by reference). Highly conserved residues include the distal His in

the E helix (E7), Phe in the CD4 region, and Pro at the beginning of the C helix (C2) (B. C. Clothia, et al., J. Mol. Biol., 196:199 (1987); S. N. Vinogradov et al., Comp. Biochem. Physiol., 106B:1 (1993), which are hereby incorporated by reference). Three of these residues (Pro in C2, Phe in CD1, His in F8) are conserved in both HemAT-*Hs* and HemAT-*Bs* (asterisks in Figure 1A). These features suggested to us that HemATs may be heme-containing proteins that generate signals in response to binding of oxygen.

Both proteins, HemAT-*Hs* and HemAT-*Bs*, can be expressed in *E. coli* from recombinant vectors. PCR primers with sequences flanking the *hemAT-Hs* gene from *H. salinarum* strain Flx15 and encoding a *Nde*I or *BamH*I restriction site were used to amplify and clone the chromosomal gene into the pET expression vector (Novogen Inc.). The PCR product was initially ligated into the pCR^R-Blunt II TOPO cloning vector (Invitrogen, Inc.) and then subcloned into pET-3b after digestion of the donor and recipient plasmids with *Nde*I and *BamH*I. The resulting plasmid was introduced into the *E. coli* pLysS strain for protein expression. PCR primers with sequences flanking the *hemAT-Bs* gene from *B. subtilis* strain OI1085 and encoding a *BamH*I or *Pst*I restriction site were used to amplify and clone the chromosomal gene into the pCR^R-Blunt II TOPO vector. This fragment was later subcloned into the pMALcII expression vector (New England Biolabs, Inc.), which was introduced into the *E. coli* pLysS strain for protein expression.

Recombinant HemAT-*Hs* is purified using anion-exchange and gel-filtration chromatography. BL21 pLysS host cells harboring plasmids carrying the *hemAT-Hs* or *hemAT-Bs* genes were grown in Luria-Bertani broth with appropriate antibiotics, and synthesis of the proteins was induced with 0.6 mM isopropyl-D-thiogalactopyranoside. After a two hour induction, the cells were harvested by low speed centrifugation (4000 x g) at 4°C for 15 min. The pellets were resuspended in buffer (50 mM NaCl, 50 mM Tris-HCl, pH 6.0) and sonicated for 4 min (12 pulses of 20 sec with 30 sec pauses). The cell lysate was centrifuged at 28,000 x g for 20 min. The red supernatant became the source of proteins for purification. The HemAT-*Hs* supernatant was applied to an anion-exchange POROS HQ/M column equilibrated with buffer (50 mM Tris-HCl, pH 6.0). A linear gradient of NaCl (0-1500 mM) was applied, and HemAT-*Hs* eluted at about 400 mM. Fractions containing HemAT-*Hs* (monitored by the Soret band absorbance at 410 nm and SDS-PAGE) were concentrated and applied to a HiLoad Superdex 200 gel-

filtration column. Fractions containing HemAT-*Hs* were concentrated with an Amicon 100K concentrator.

A saturated $(NH_4)_2S0_4$ solution was added to 30% saturation to the HemAT-*Bs* supernatant and centrifuged at 28,000 x g for 20 min. The optically clear, light-red supernatant was further fractionated by adding $(NH_4)_2S0_4$ to 36% saturation, and the precipitate was pelleted by centrifugation. The pellet was resuspended in buffer (200 mM NaCl, 50 mM Tris-HCl, pH 8.0) and applied to a HiLoad Superdex 75 column. Fractions containing HemAT-*Bs* were concentrated with an Amicon 50K concentrator.

During SDS-polyacrylamide gel electrophoresis (SDS-PAGE), purified HemAT-*Hs* migrates slower than expected from its calculated molecular mass of 52.8 kDa (Figure 2A, lane 1). This behavior is consistent with the highly acidic nature of many halophilic proteins (K. Ihara et al., Arch. Biochem. Biophys., 286:111 (1991), which is hereby incorporated by reference). HemAT-*Hs* is purified from *H. salinarum* by metal-affinity and gel-filtration chromatography as a recombinant protein ($HemAT_{6xHis}$-*Hs*) carrying a carboxyl-terminal six-histidine tag (Figure 2A, lane 2). A plasmid encoding carboxyl-terminal 6 His-tagged HemAT-*Hs* was constructed by two-step PCR. In the first step, 6 His codons were fused to *hemAT-Hs* immediately in front of the natural stop codon. In the second step, a *Xba*I restriction site was introduced at the 3' end of the gene. The second PCR product was subcloned into the *Nde*I and *Xba*I sites of plasmid pKJ427. This plasmid was introduced into a Δ*hemAT-Hs* strain (A. Brooun et al., J. Bacteriol., 180:1642 (1998), which is hereby incorporated by reference). Cells grown at 39 °C to mid-logarithmic phase were harvested by centrifugation (4000 x *g*) at 4 °C. The pellet was resuspended in buffer (200 mM NaCl, 50 mM Tris-HCl, pH 8.0) and sonicated for 3 min (12 pulses of 15 sec with 20 sec pauses). The cell lysate was centrifuged (100,000 x *g*) at 14 °C for 30 min, and the supernatant was used for purification. The POROS MC/M affinity column was washed with 1 M NaCl, 50 mM EDTA (pH 8.0), charged with 100 mM $CoCl_2$, and finally washed with 3M NaCl. The column was equilibrated with buffer (200 mM NaCl, 50 mM Tris-HCl, pH 8.0) prior to loading the sample. $HemAT_{6xHis}$-*Hs* was eluted with a linear gradient of imidazole (0-250 mM). The peak fractions were collected, concentrated, and applied to a HiLoad Superdex 200 gel-filtration column. The peak fractions were concentrated with an Amicon 100K concentrator. HemAT-*Bs* is purified using a combination of ammonium-sulfate precipitation/fractionation and gel-

filtration chromatography. As expected, purified HemAT-*Bs* migrates during SDS-PAGE as a 48.7 kDa protein (Figure 2A, lane 3).

The preferred bacterial heme binding proteins are myoglobin-like proteins. In particular, the heme binding proteins show greater than 20% identity to a vertebrate myoglobin protein, such as sperm whale myoglobin. More preferred are proteins which show greater than 30% or 50% identity. The level of identity is calculated using the protein alignment program of BLAST with the default parameters.

In a preferred embodiment, the heme-binding protein is isolated from *Archaea*. The *Archaea* are a group of organisms often found in extreme environments, such as high temperatures, high salt concentrations, and acidic conditions. The conditions are often so extreme that other organisms are unable to survive in that environment. Proteins isolated from the *Archaea* often exhibit higher stability in the presence of high temperatures, high salt concentrations, or low pH. Generally, the proteins isolated from *Archaea* are preferred due to their higher stability.

In particular, the protein is isolated from *Halobacterium salinarum*. *H. salinarum* is a salt tolerant organism. Similarly, the HemAT-*Hs* protein is salt tolerant. The sequence of the gene encoding the HemAT-*Hs* protein is shown in SEQ. ID. No. 1 as follows:

```
ATGAGCAACG ATAATGACAC TCTCGTGACC GCCGACGTTC GGAACGGGAT CGACGGGCAC   60
GCACTCGCGG ACCGGATCGG CCTCGACGAG GCGGAGATCG CGTGGCGGCT GTCGTTCACC  120
GGGATCGACG ACGACACGAT GGCCGCGCTC GCCGCCGAAC AGCCGCTGTT CGAAGCCACC  180
GCGGACGCGC TGGTGACCGA CTTCTACGAC CACTTGGAGT CCTACGAGCG CACACAGGAC  240
CTCTTCGCGA ACTCCACGAA GACCGTCGAG CAACTCAAAG AGACGCAGGC CGAGTACTTG  300
CTGGGCCTCG GGCGCGGCGA GTACGACACC GAGTACGCCG CCCAGCGCGC CCGTATCGGG  360
AAGATACACG ACGTGCTCGG GCTCGGACCG GACGTCTATC TGGGCGCGTA CACGCGATAC  420
TACACGGGGC TGTTGGACGC GCTTGCCGAC GACGTGGTCG CCGACCGCGG CGAGGAGGCG  480
GCCGCCGCCG TCGACGAACT CGTGGCCCGG TTCCTGCCGA TGTTGAAGCT GTTGACCTTC  540
GATCAGCAGA TCGCAATGGA CACCTACATC GACTCGTACG CCCAGCGCCT CCACGACGAG  600
ATCGACAGCC GCCAGGAGTT GGCGAACGCG GTCGCCACGC ACGTGGAAGC ACCGCTGTCC  660
TCGCTGGAGG CGACCTCGCA GGACGTCGCC GAGCGCACGG ACACGATGCG GGCCCGCACC  720
GACGACCAGG TCGACCGCAT GGCTGACGTC AGCCGTGAGA TATCCAGCGT GTCCGCGAGC  780
GTCGAGGAGG TCGCCTCGAC GGCCGACGAC GTCCGCCGGA CCAGCGAGGA CGCCGAGGCG  840
CTGGCCCAGC AGGGCGAGGC GGCCGCCGAC GACGCGCTCG CCACGATGAC CGACATCGAC  900
GAGGCGACCG ACGGCGTCAC CGCGGGCGTC GAACAGCTCG GCGAGCGCGC CGCCGACGTC  960
GAATCAGTGA CCGGCGTGAT CGACGACATC GCCGAGCAGA CGAACATGCT GGCGCTGAAC 1020
GCGTCCATCG AGGCCGCCCG CGCCGGGGAG GCGGGCGAGG GGTTTGCGGT CGTCGCCGAC 1080
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GAGGTCAAGG CCCTCGCCGA GGAGTCCCGC GAGCAGTCCA CGCGCGTCGA GGAGCTCGTC 1140
GAGCAGATGC AGGCGGAGAC CGAGGAGACG GTCGACCAGT TGGACGAGGT CAACCAGCGC 1200
ATCGGCGAGG GCGTCGAGCG CGTCGAGGAG GCGATGGAGA CCCTCCAGGA GATCACCGAC 1260
GCCGTCGAGG ACGCCGCAAG CGGGATGCAG GAGGTGTCGA CGGCGACCGA CGAACAGGCG 1320
GTGAGCACCG AGGAGGTCGC CGAGATGGTC GACGGTGTCG ACGACCGCGC GGGCGAGATC 1380
GCGGCCGCCC TCGATGACAT CGCCGACGCG ACCGATCAGC AGGTCCGGAC CGTCGAGGAG 1440
GTCCGCGAGA CGGTCGGCAA GCTCAGCTAG                                   1470
```

The *hemAT-Hs* gene encodes a protein which has an amino acid sequence as shown in SEQ. ID. No. 2 as follows:

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MSNDNDTLVTADVRNGIDGHALADRIGLDEAEIAWRLSFTGIDDDTMAALAAEQPLFEAT   60
ADALVTDFYDHLESYERTQDLFANSTKTVEQLKETQAEYLLGLGRGEYDTEYAAQRARIG  120
KIHDVLGLGPDVYLGAYTRYYTGLLDALADDVVADRGEEAAAAVDELVARFLPMLKLLTF  180
DQQIAMDTYIDSYAQRLHDEIDSRQELANAVATHVEAPLSSLEATSQDVAERTDTMRART  240
DDQVDRMADVSREISSVSASVEEVASTADDVRRTSEDAEALAQQGEAAADDALATMTDID  300
EATDGVTAGVEQLGERAADVESVTGVIDDIAEQTNMLALNASIEAARAGEAGEGFAVVAD  360
EVKALAEESREQSTRVEELVEQMQAETEETVDQLDEVNQRIGEGVERVEEAMETLQEITD  420
AVEDAASGMQEVSTATDEQAVSTEEVAEMVDGVDDRAGEIAAALDDIADATDQQVRTVEE  480
VRETVGKLS                                                      489
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In another embodiment of the invention, the heme-binding protein is isolated from *Bacillus subtilis.* Preferably, the *Bacillus subtilis* gene is *hemAT-Bs*, which has a nucleic acid sequence according to SEQ. ID. No. 3, as follows:

```
ATGTTATTTA AAAAAGACAG AAAACAAGAA ACAGCTTACT TTTCAGATTC AAACGGACAA   60
CAAAAAAACC GCATTCAGCT CACAAACAAA CATGCAGATG TCAAAAAACA GCTCAAAATG  120
GTCAGGTTGG GAGATGCTGA GCTTTATGTG TTAGAGCAGC TTCAGCCACT CATTCAAGAA  180
AATATCGTAA ATATCGTCGA TGCGTTTTAT AAAAACCTTG ACCATGAAAG CTCATTGATG  240
GATATCATTA ATGATCACAG CTCAGTTGAC CGCTTAAAAC AAACGTTAAA ACGGCATATT  300
CAGGAAATGT TTGCAGGCGT TATCGATGAT GAATTTATTG AAAAGCGTAA CCGAATCGCC  360
TCCATCCATT TAAGAATCGG CCTTTTGCCA AAATGGTATA TGGGTGCGTT TCAAGAGCTC  420
CTTTTGTCAA TGATTGACAT TTATGAAGCG TCCATTACAA ATCAGCAAGA ACTGCTAAAA  480
GCCATTAAAG CAACAACAAA AATCTTGAAC TTAGAACAGC AGCTTGTCCT TGAAGCGTTT  540
CAAAGCGAGT ACAACCAGAC CCGTGATGAA CAAGAAGAAA AGAAAAACCT TCTTCATCAG  600
AAAATTCAAG AAACCTCTGG ATCGATTGCC ATTCTGTTTT CAGAAACAAG CAGATCAGTT  660
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CAAGAGCTTG TGGACAAATC TGAAGGCATT TCTCAAGCAT CCAAAGCCGG CACTGTAACA  720
TCCAGCACTG TTGAAGAAAA GTCGATCGGC GGAAAAAAAG AGCTAGAAGT CCAGCAAAAA  780
CAGATGAACA AAATTGACAC AAGCCTTGTC CAAATCGAAA AAGAAATGGT CAAGCTGGAT  840
GAAATCGCGC AGCAAATTGA AAAAATCTTC GGCATCGTCA CAGGCATAGC TGAACAAACA  900
AACCTTCTCT CGCTCAATGC ATCTATTGAA TCCGCCCGCG CCGGAGAACA CGGCAAAGGC  960
TTTGCTGTCG TGGCAAATGA AGTGCGGAAG CTTTCTGAGG ATACGAAAAA AACCGTCTCT 1020
ACTGTTTCTG AGCTTGTGAA CAATACGAAT ACACAAATCA ACATTGTATC CAAGCATATC 1080
AAAGACGTGA ATGAGCTAGT CAGCGAAAGT AAAGAAAAAA TGACGCAAAT TAACCGCTTA 1140
TTCGATGAAA TCGTCCACAG CATGAAAATC AGCAAAGAGC AATCAGGCAA AATCGACGTC 1200
GATCTGCAAG CCTTTCTTGG AGGGCTTCAG GAAGTCAGCC GCGCCGTTTC CCATGTGGCC 1260
GCTTCCGTTG ATTCGCTTGT CATCCTGACA GAAGAATAAC CATCAAAAAC CGGTCTGCCA 1320
TACGGCCGGT TTTTTTGCGT TCATTATGTA AACTTAAATT AAAAATCAGT TGACATAATA 1380
ATTACCTGCA                                                         1390
```

In a preferred embodiment, the protein has an amino acid sequence of SEQ. ID. No. 4, as follows:

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MLFKKDRKQETAYFSDSNGQQKNRIQLTNKHADVKKQLKMVRLGDAELYVLEQLQPLIQE   60
NIVNIVDAFYKNLDHESSLMDIINDHSSVDRLKQTLKRHIQEMFAGVIDDEFIEKRNRIA  120
SIHLRIGLLPKWYMGAFQELLLSMIDIYEASITNQQELLKAIKATTKILNLEQQLVLEAF  180
QSEYNQTRDEQEEKKNLLHQKIQETSGSIANLFSETSRSVQELVDKSEGISQASKAGTVT  240
SSTVEEKSIGGKKELEVQQKQMNKIDTSLVQIEKEMVKLDEIAQQIEKIFGIVTGIAEQT  300
NLLSLNASIESARAGEHGKGFAVVANEVRKLSEDTKKTVSTVSELVNNTNTQINIVSKHI  360
KDVNELVSESKEKMTQINRLFDEIVHSMKISKEQSGKIDVDLQAFLGGLQEVSRAVSHVA  420
ASVDSLVILTEE                                                   432
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The invention also provides fragments of the isolated heme-binding protein which contain a functional heme-binding domain. The fragment containing the functional heme-binding domain may be coupled to a heterologous signal transduction domain. As described in the examples, a minimum heme binding domain has been determined for HemAT-*Hs* and partially determined for HemAT-*Bs*. Furthermore, comparisons

beteween various globin proteins has allowed for the identification of conserved regions of the proteins.

HemAT-*Hs* in *Halobacterium salinarum* and HemAT-*Bs* in *Bacillus subtilis*, the first aerotactic transducers discovered that directly bind oxygen, are heme-based, and are homologous to native sperm whale myoglobin (SWMb), albeit more structural than sequential. These proteins belong to the globin family. Globins bind, transport, and store oxygen, and are known to exhibit a distinctive fold of seven α-helices that encompass a heme prosthetic group. The seven helices are labeled A, B, C, E, F, G, and H. Sometimes, an additional short helix (helix D) is found between helices C and E, as in the case of SWMb, to make a total of eight. In a 1987 publication, Bashford et al., "Determinants of a Protein Fold: Unique Features of the Globin Amino Acid Sequences," J. Mol. Biol., 196:199-216 (1997), which is hereby incorporated by reference, reported that the sequence homology of all the 226 globin sequences known at that time were "as high as 80% or more for closely related species, or as low as 16% for more distant ones." Of all these proteins, only two residues were absolutely conserved throughout. These two residues were the phenylalanine at the end of the C helix (CD1) and the proximal histidine (F8). HemAT-*Hs* and HemAT-*Bs* both contain these two key residues and are 23% and 11% homologous to SWMb, respectively, and share 20% sequence similarity between themselves.

The report of myoglobin-type aerotaxis proteins in microorganisms, and the recent discovery of HemAT-*Hs* and HemAT-*Bs* has prompted an effort to find one or more signature motifs in these possible microbial globins. These would identify conserved regions of the proteins. In addition, with these motifs in hand, contemporary computer algorithms like those contained in the BLAST programs (http://www.ncbi.nlm.nih.gov/BLAST/) could permit convenient and rapid searches for other possible globins using this signature motif. These motifs could be used for classifying these newly discovered microbial globins together and eventually with the whole globin family.

Vianogradov et al., "Adventitious Variability? The Amino Acid Sequences of Nonvertebrate Globins," Comp. Biochem. Physiol., 106B:1-26 (1993), which is hereby incorporated by reference, have noted the extensive variation of invertebrate globins over the vertebrates and Bashford et al., "Determinants of a Protein Fold: Unique Features of the Globin Amino Acid Sequences," J. Mol. Biol., 196:199-216 (1987), which is hereby

incorporated by reference, have recognized that alignments of invertebrate globins with vertebrate globins based strictly on sequence similarity and vertebrate data sets are questionable. Invertebrate myoglobins were therefore not included in the preliminary data and the search for a globin motif was limited to vertebrates. Microbial globins, however, were later included and incorporated into the alignment by conserving secondary structure and avoiding gaps as in the work of Kapp et al., "Alignment of 700 Globin Sequences: Extent of Amino Acid Substitution and Its Correlation With Variation in Volume," Pro. Sci., 4:2179-2190 (1995), which is hereby incorporated by reference.

An 80-aa consensus peptide sequence was constructed using the manual alignment of sperm whale myoglobin (SWMb), the oxygen sensor in *Bacillus subtilis*, HemAT-*Bs*, and the oxygen sensor in *Halobacterium salinarum*, HemAT-*Hs*. The intent was to find a minimal length of protein containing the myoglobin signature motif and see how many myoglobin proteins this sequence would recognize on the non-redundant (nr) database at NIH using the BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/). An X was issued to residues of high variability (Bashford et al., "Determinants of a Protein Fold: Unique Features of the Globin Amino Acid Sequences," J. Mol. Biol., 196:199-216 (1987), which is hereby incorporated by reference) while conserved residues retained their specific amino acid designation. Critical to the alignment was the positioning of the two residues known to be absolutely conserved in all known globins: Phe at the CD1 position and the proximal His at the F8 position (Bashford et al., "Determinants of a Protein Fold: Unique Features of the Globin Amino Acid Sequences," J. Mol. Biol., 196:199-216 (1987), which is hereby incorporated by reference). Using these residues as markers, the myoglobin-like protein (MbLP) sequence was generated and consisted of two domains separated by 32 variable amino acids. The first myoglobin-type domain (M1-box) contained the absolutely conserved phenylalanine residue; the second (M2-box) contained the absolutely conserved proximal histidine. A BLAST search was then performed, comparing the sequences of MbLP and SWMb with those of all other proteins in the non-redundant database. Search parameters were default except for the EXPECT parameter, which was increased to 1000 to allow for matches of lesser sequence homology. This comparison between the number and type of SWMb hits and MbLP hits was used to assess the quality of the MbLP sequence in extracting myoglobin proteins.

A microbial globin-type sequence was generated from the results of a previous BLAST search on microbial globins and included *Vitreoscilla* hemoglobin for structural

markers. This sequence was used to extract 9 bacterial and 8 eukaryotic hemoglobins and flavohemoproteins. This sequence was generated to incorporate microbial globins into the search of a combined globin motif. Manipulation and alignment of the microbial globin-type peptide with MbLP and incorporating the same marker residues produced a second consensus sequence 96-amino acids in length called the triplet globin motif (TGM) because it consisted of three domains: two myoglobin-type domains (M1-box, M2-box) and one hemoglobin-type domain (H-box). TGM was the final sequence used for further analysis and BLASTP searches with the TGM sequence were performed at a lower EXPECT parameter of 600 to reduce the amount of false-positives.

The ability of the myoglobin motif to recognize myoglobins was tested using SWMb as a reference. A BLASTP search of the non-redundant protein database was performed using the 153-aa native sperm whale myoglobin (SWMb) as the query sequence. This sequence recognized 83 unique myoglobins and a wealth of hemoglobins. With some manipulation of the search conditions, however, SWMb was able to extract HemAT-*Hs* as well.

A first attempt at a globin-type motif produced the 80-aa myoglobin-like protein (MbLP) sequence consisting of two domains, the M1-box and M2-box, as found in Figure 3. These two domains recognized 73 myoglobins, or 88% of those found by SWMb, along with HemAT-*Hs*, HemAT-*Bs*, and a few non-globins. In contrast, however, MbLP didn't recognize any hemoglobins.

An effort was made to enhance the globin-type motif of MbLP by building upon itself. This effort resulted in the 96-aa triplet globin motif (TGM) protein sequence and consisted of three domains: the M1-, M2-box, and a new H-box situated in front of the two. The TGM sequence was compared to the MbLP and SWMb by subjecting it to the same BLASTP search analysis. TGM recognized 75 myoglobins (90% of SWMb hits), 17 hemoglobin and hemoglobin-like proteins, and the two HemATs. The 17 hemoglobin and hemoglobin-like proteins consisted of 5 non-microbial eukaryotic hemoglobins from three different organisms and 12 microbial hemoglobins, three eukaryotic and nine bacterial. It is evident that the TGM sequence is more general than MbLP in recognizing globin motifs.

Table 1. Alignment and classification of some of the resultant proteins in the H-box and M1-box region using TGM as the template. Shaded residues are conserved in their respective box (H-, M1-, M2-box). Bold residues are highly conserved in their box.

Kingdom	Globin	Secondary (SWMb ->)			HHHHHHHHHHtHHHHHHHHHHHHHHHHhhhhhhhhhlllllllllllHHHHHHHHhhhhhhhh A >| |< B >|< C >| |< D >|< E |< H Box >|< . M1 Box >|			
		TEMPLATE	tmpseq_1	1	IIKXTVPVLXEHGXXIGQDVLVVLIKXNPEIQ-EKF--FFFKHXXXXXXXXXXXXXXXXX	57	SEQ. ID. No.	5
B	H	HMPA_ERWCH	Q47266	10	IKSTIPLLAETGPALTAHFYQRMFHHNPELK-DIF--NMSNQRNGDQREALFNAICAY	64	SEQ. ID. No.	6
B	H	BAHG_VITST	P04252	9	IIKATVPVLKEHGVTITTTFYKNLFAKHPEVR-PLF--DMGRQESLEQPKALAMTVLAA	64	SEQ. ID. No.	7
B	H	HMPA_ECOLI	P24232	10	VKATIPLLVETGPKLTAHFYDRMFTHNPELK-EIF--NMSNQRNGDQREALFNAIAAY	64	SEQ. ID. No.	8
B	H	FHG_SMNLA	2738912	10	VKATIPLLVETGPKLTAHFYDRMFTHNPELK-EIF--NMSNQRNGDQREALFNAIAAY	64	SEQ. ID. No.	9
B	H	HMPA_ALCEU	P39662	9	IVKATAPVLAEHGYDIIKCFYQRMFEAHPELK NVF--NMAHQEQGQQQQALARAVYAY	64	SEQ. ID. No.	10
B	H	HMPA_VIBPA	P40609	9	IVKATAPLIAETGPKLTAHFYDRMFTHNPELK-DIF--NMSNQRNGDQREALFNAICAY	64	SEQ. ID. No.	11
B	H	BAHG_CLOS	BAA81644	9	IIKSTVPVLKSNGLEITKTFYKNMFEQNPEVK-PLF--NMNKQESEEQPKALAMAILAV	64	SEQ. ID. No.	12
E	H	FHG_FUOXY	BAA33011	10	IVKSTAPILKEHGKTITTTFYRNMLGAHPELK-NYF--SLRNQQTGAQQAALANSVLAY	65	SEQ. ID. No.	13
B	H	FHP_AQUAE	2982927	9	VIKSTVPLLKEHGTEITARMYELLFSKYPKTK-ELF--AGA---SEEQPKKLANAIIAY	61	SEQ. ID. No.	14
B	H	HMPA_BACSU	P49852	9	IIKSTVPVLQQHGETITGRFYDRMFQDHPELL-NIF--NQTNQKKKTQRTALANAVIAA	64	SEQ. ID. No.	15
E	H	HGA1_XENO	CAA32474	11	IKAIMPSIAAHGDTFGGEALYRMFLVNPKTK-TYFPSFDFHHNSK-QITSHGKKVVDA	66	SEQ. ID. No.	16
E	H	GLB8_CHITH	P02227	8	DQLALFKSSWNTVKHNEVDILYAVFKANPDIQ-AKFPQFAGKDLDSIKDSADF-AVHSG	64	SEQ. ID. No.	17
E	H	HBA1_XENBO	P07430	11	IKAIMPSIAAHGDKFGGEALYRMFLVNPKTK-TYFPTFDFHHNSK-QISAHGKKVVDA	66	SEQ. ID. No.	18
E	H	HBA2_XENBO	P07431	11	IKAILPSIAAHGDKFGGEALYRMFLINPKTK-TYFPNFDFHHNSK-QISAHGKKVVDA	66	SEQ. ID. No.	19
E	H	GLB11_CHIT	2155298	24	QAILIRSSWEDEVKHNEVDILYAIFKANPDIQ-ARFPQFAGKDLDSIKTTGQF-AVHAG	79	SEQ. ID. No.	20
E	H	FHP_CANNO	Q03331	21	LQSLAPVVKEHGVTVTSTMYKYMFQTYPEVR-SYF--NMTNQKTGRQPKVLAFSLYQY	75	SEQ. ID. No.	21
E	H	FHG_YEAST	S57699	9	IIKATVPVLEQQGTVITRTFYKNMLTEHTELL-NIF--NRTNQKVGAQPNALATTVLAA	64	SEQ. ID. No.	22
E	M	MYG_PHYCA	P02185	25	GQDILIRLFKSHPETL-EKF--DRFKHLKTEAEMKASEDLKKHG	65	SEQ. ID. No.	23
E	M	MYG_KOGSI	P02184	25	GQDILIRLFKHHPETL-EKF--DRFKHLKSEAEMKASEDLKKHG	65	SEQ. ID. No.	24
E	M	MYG_ROUAE	P02163	25	GQEVLIRLFKGHPETL-EKF--DKFKHLKSEDEMKASEDLKKHG	65	SEQ. ID. No.	25
E	M	MYG_TURTR	P02172	25	GQDVLIRLFKGHPETL-EKF--DKFKHLKTEADMKASEDLKKHG	65	SEQ. ID. No.	26
E	M	MYG_GLOME	P02174	25	GQDILIRLFKGHPETL-EKF--DKFKHLKTEADMKASEDLKKHG	65	SEQ. ID. No.	27
E	M	MYG_WHAUK	JT0636	25	GHQVLMRLFQDHPETL-DRF--DKFKGLKTPDQMKGSEDLKKHG	65	SEQ. ID. No.	28
E	M	MYG_MUSAN	P14399	21	GQNILLRLFEQYPESQ-NHF--PKFKN--KSLGELKDTADIKAQ	59	SEQ. ID. No.	29

Table 2 Alignment and classification of some of the resultant proteins in the M2-box region using TEMPLATE as the template. Shaded residues are conserved in their respective boxes (H-, M1-, M2-box).

Kingdom	Globin	Secondary			hhhhhhhhhhhhlllllllllHHHHHHHHHHHHlllllhhhhh E >| |< F >| |< G |< M2 Box >|	
		TEMPLATE	tmpseq_1	58	XXXXXXXXXXXXXXXXXXAQRXR-LAQIHAXKGKIPDWYL	96
					SEQ. ID. No. 30	
E	M	MYG_PHYCA	P02185	66	VTVLTALGAILKKKGHHEAELKP-LAQSHATKHKIPIKYL	104
					SEQ. ID. No. 31	
E	M	MYG_KOGSI	P02184	66	VTVLTALGAILKKKGHHEAELKP-LAQSHATKHKIPIKYL	104
					SEQ. ID. No. 32	
E	M	MYG_ROUAE	P02163	66	ATVLTALGGILKKKGQHEAQLKP-LAQSHATKHKIPVKYL	104
					SEQ. ID. No. 33	
E	M	MYG_TURTR	P02172	66	NTVLTALGAILKKKGHHDAELKP-LAQSHATKHKIPIKYL	104
					SEQ. ID. No. 34	
E	M	MYG_GLOME	P02174	66	NTVLTALGAILKKKGHHEAELKP-LAQSHATKHKIPIKYL	104
					SEQ. ID. No. 35	
E	M	MYG_WHAUK	JT0636	66	VTVLTQLGKILKQKGNHESELKP-LAQTHATKHKIPVKYL	104
					SEQ. ID. No. 36	
B	M?	HemAT-*Bs*	CAA74545	96	LKRHIQEMFAGVIDDEFIEKRNR-IASIHLRIGLLPKWYM	134
					SEQ. ID. No. 37	
E	M	MYG_MUSAN	P14399	60	ADTVLSALGNIVKKKGSHSQPVKALAATHITTHKIPPHYF	99
					SEQ. ID. No. 38	
A	M?	HemAT-*Hs*	1654421	96	QAEYLLGLGRGEYDTEYAAQRAR-IGKIHDVLGLGPDVYL	134
					SEQ. ID. No. 39	

The secondary structure reported in Figure 3 is that of SWMb and is considered typical of the globins. It was interesting to note that the domain responsible for HemAT recognition, the M2-box, lies in the region between the F and G helix, which contains the proximal histidine. Alignments indicate that two loop regions of the HemATs (CD and EF loops) are much more extensive than in SWMb. The M2-box does not include the HemATs' distinctive EF loop, thereby allowing recognition of both of the transducers. The M1-box not only includes the B and C helix, but also specifies the entire CD loop region, which, inadvertently, ends up excluding the HemATs.

The H-box recognizes primarily microbial hemo-globins/proteins, which is equivalent in position to the last two-thirds of helix A and the first third of helix B of SWMb. This region is highly significant, as it is could help place a sequence like TEMPLATE in an phylogenetic tree, thereby connecting the eukaryotic and eubacterial hemoglobins with the myoglobins and myoglobin-like proteins. The M1-box, containing what would be helices B and C from SWMb, incorporates one of the absolutely conserved residues, Phe, from the CD region and only pulls out myoglobins from higher species. Though the match scores are much lower, the M2-box pulls out almost the same myoglobins as the M1-box, however, recognition of HemAT-*Hs* and HemAT-*Bs* occurs only in the M2-box.

Based upon the extensive information available regarding conserved structures in the proteins, as well as the minimal functional regions, one can predict modifications to the proteins which will not alter the function of the protein.

The present invention also provides a blood substitute. An urgent need exists among the medical community for an alternative to whole blood or red blood cells for use in transfusion. However, the possibility of transmitting viral infections is ever present in derivatives of human blood. The rapid spread of the AIDS virus as well as the discovery of multiple forms of the virus amplifies this concern. Both HemAT-*Hs* and/or HemAT-*Bs* may present an alternative to whole blood in transfusion situations. HemAT-*Hs* and HemAT-*Bs* are particularly attractive in this regard, since they appear to have low oxygen affinity, a property required for artificial oxygen carriers. Expressed in microorganisms, these oxygen carriers will be free of infectious agents. In addition, the ability of HemAT-*Hs* and HemAT-*Bs* to bind oxygen reversibly and to regulate this binding through the signal transduction domain may lead to new blood substitute products. Currently cross-linked hemoglobins are being developed as blood substitutes but these proteins suffer from poor regulation of oxygen binding. It is possible to develop HemAT-*Hs* and HemAT-*Bs* as a "blood-substitute" due to their size (~50 kDa, similar to hemoglobin) which prevents filtering by the kidney's, its ability to reversibly bind oxygen, and its ability to regulate this binding. Genetically engineered fragments of the *hemAT-Hs* and *hemAT-Bs* genes that encode of the transduction domain provides a wealth of opportunities to regulate oxygen binding.

In a preferred embodiment, the blood substitute has a heme binding domain of the isolated heme-binding protein. The blood substitute may also have a heterologous signal transduction domain, to alter the affinity for oxygen or other gases.

The blood substitute may be administered to a patient suffering from low blood levels. Such a blood substitute has numerous advantages because it could be used as a substitute when whole blood is not available. Furthermore, the blood substitute can be produced so that it is free of infectious substances, such as viruses and bacteria.

In addition to using heterologous signaling domains, the oxygen binding of the heme-binding protein may be altered by modifying the signaling domain.

The invention also provides a method for controlled storage of oxygen. The bacterial heme binding protein can be contacted with oxygen allowing the protein to bind and store oxygen. The protein may also be covalently attached to a solid substrate via the

transduction domain. Subsequent triggering of the transduction domain can result in oxygen release.

The present invention may also be used to sense gaseous ligands by exposing the bacterial heme binding protein to a sample to be tested and measuring a change in the conformation of the protein. Enzyme sensors are well known as biological sensors. They are utilized mainly for clinical chemical analysis, including use for glucose in blood, urea and neutral and phospholipids. The ability of HemAT-*Hs* and HemAT-*Bs* to sense oxygen as well as other small gaseous ligands provides opportunities to develop novel biosensors for O_2, NO, CO, and even CN^-.

The changes in the conformation of the protein may be monitored in various ways including monitoring the protein optically or electronically.

The preferred gaseous ligands to monitor with the heme binding bacterial protein are O_2, NO, CO, and CN^-. The preferred gaseous ligand is O_2.

As discussed above fragments of the bacterial heme binding protein may also be used as long as they contain a functional heme binding domain.

The present invention also provides a chimeric protein having a heme-binding domain of an isolated heme binding bacterial protein and a heterologous signaling domain. Varying the signaling domain can alter the oxygen or ligand binding characteristics of the protein. The signaling domain may also be altered to make the protein responsive to other signals.

In another embodiment, the invention provides an isolated nucleic acid molecule which encodes a bacterial heme binding protein with a heterologous or mutated signaling domain.

The bacterial heme-binding proteins may also be used for heme-based catalysis. It is well known that Fe(III)porphyrins can catalyze a wide variety of chemical reactions including hydrogen peroxide degradation, mono oxygenation, and lignin degradation. HemAT-*Hs* can also be prepared in the Fe(III) form providing an opportunity to utilize this protein as a novel heme-based catalyst. In addition, the ability to regulate the heme domain by the transduction domain may allow for catalytic specificity to be achieved via genetic manipulation of this domain.

The proteins of the present invention may also be used for artificial photosynthesis. HemAT-*Hs* can be reconstituted with different porphyrins including photoactive Zn and Sn derivatives. These derivatives may posses the ability to absorb

light energy and transmit information concerning the excited state of the photoactive poprhyrin to the sensing domain providing-the equivalent to photosynthesis, i.e., conversion of light energy to chemical potential energy.

The bacterial heme binding proteins may also be used in *in vivo* and *in vitro* testing system for identifying potential signaling functions of mutated a-hemoglobin and myoglobin causing several diseases. Mutated human α-hemoglobin and myoglobin genes can be fused with fragment of *hemAT-Hs* or *hemAT-Bs* genes that encodes signaling domain via linker region. The physiological function of the expressed chimeric protein of human α-hemoglobin (or myoglobin) and HemAT-*Hs* or HemAT-*Bs* can be tested by capillary aerotaxis assay. As transducer proteins HemAT-*Hs* and HemAT-*Bs* may cause phosphorylation of CheA. Once this feature of HemAT-*Hs* and HemAT-*Bs* are tested and optimized, similar *in vivo* strategy of chimeric protein construction can be tested for *in vitro* phosphorylation assay.

EXAMPLES

Example 1 – Mutagenesis of HemAT-*Hs* and HemAT-*Bs*

The HtrVIII is a positive aerotaxis transducer in *H. salinarum* (Brooun et al., J. Bacteriol., 180:1642-1646 (1998), which is hereby incorporated by reference). A strain deleted for the *htrVIII* gene lacks positive aerotaxis while a strain overproducing the protein shows an enhanced aerotactic response. To investigate the possible role of HemAT-*Hs* and HemAT-*Bs* in aerotaxis, deletion mutants of these genes were constructed (Brooun, Ph.D thesis. University of Hawaii, Hawaii (1997), which is hereby incorporated by reference) for the construction of *hemAT-Hs* deletion strains. Construction of overexpression of *hemAT-Hs* in *H. salinarum*: *Nde*I and *Xba*I restriction sites were used to clone the *hemAT-Hs* gene into the *E. coli-H. salinarum* shuttle vector pKJ427. Top primer with *Nde*I cutting site (5'CCGAATTCCATATGAGCAACGAT AATGAC 3' (SEQ. ID. No. 40)) and bottom primer with *Xba*I cutting site (5'CCTCTA GAGGATEECTAGCTGAGCTTGCCGACC 3' (SEQ. ID. No. 41)) were synthesized and used for PCR amplification of *hemAT-Hs* gene. The PCR amplicon was cloned into TOPO cloning vector (Invitrogen) and transformed into *E. coli* competent cells. The plasmid containing *hemAT-Hs* gene in TOPO vector was subcloned into pKJ427 vector

by *NdeI/XbaI* double digestion. The *hemAT-Hs*/pKJ427 construction was confirmed by PCR as well as *NdeI/XbaI* double digestion and transformed into Δ*htrVIII* strain using standard halobacteria transformation protocol. Individual colonies were checked by PCR and immunoblot to confirm the expression level of HemAT-*Hs*; Construction of OI3428: A 322 bp fragment interior to HemAT-*Bs* was amplified from the *B. subtilis* wild type strain OI1085 chromosome using primers with overhanging *Hind*III and *Bam*H1 sites (reverse primer: 5' TATGGGATCCCTTGTTCATCACGGGTCTETTGG 3' (SEQ. ID. No. 42), forward primer: 5' GATAAAGCTTGATCATAGCTCAGTTGACCG 3' (SEQ. ID. No. 43)). This PCR fragment was digested with *Hind*III and *Bam*H1 and cloned in the integration vector pHV501 (Vagner et al., Microbiology, 144(Pt 11):3097-3104 (1998)) to create pMK1. The resultant plasmid pMK1 was transformed into OI1085 and HemAT-*Bs* mutants were selected by erythromycin resistance. Integration of the pMKI into the correct locus was checked by linkage analysis. The *hemAT-Bs* locus is 30% linked to the *glyk* locus as determined from the *B. subtilis* chromosomal map. GLY+ transductants were selected and scored for erythromycin resistance. Construction of OI3498: The entire HemAT-*Bs* gene including the native promoter and the ribosome binding site was amplified from the *B. subtilis* wild type strain OI1085 chromosome using primers with overhanging *EcoR*I and *BamH*I sites (HemAT-*Bs* amyup: 5' TGCTGAATTCGCAGCTTTCATTCATGTTTCCC 3'(SEQ. ID. No. 44), HemAT-*Bs* amydown: 5' TTAGGGATCCGTCAACTGATTTTTAA TTTAAGTTAC 3') (SEQ. ID. No. 45)). The PCR amplicon was digested with *EcoRI/BamHI* and cloned into the amyE integration vector pDG1730 (Guerout-Fleury et al., Gene, 180(1-2):57-61 (1996), which is hereby incorporated by reference) to produce pKZ2. The resultant plasmid pKZ2 was digested with *BglI/Xba*I to ensure a double crossover event into the *amyE* locus and then transformed into OI3428 to select for Spec-R. HemAT-*Bs* overexpression R4: Overexpression construction in *E. coli*: The HemAT-*Bs* overexpression construction was performed as follows: *B. subtilis* OI1085 genomic DNA was used for the PCR amplification of HemAT-*Bs* gene by Pfu DNA polymerase using two primers (Top primer with *BamH*I restriction site: 5'ATATGGATCC AAGGGGGATCATTGTAATGTTATTTAAAAAAG3' (SEQ. ID. No. 46), Bottom primer with *Pst*I site: 5' ATTACTGCAGCAACTGATTTTTAATTTAAGTTT ACATAATGAACGC 3' (SEQ. ID. No. 47)). The PCR amplicon was cloned into TOPO cloning vector (Invitrogen) and transformed into TOP 10 *E. coli* competent cells.

Colonies were tested for the presence of plasmids containing the correct insert. The recombinant plasmid was digested with *BamH*I and *Pst*I and the insert with HemAT-*Bs* open reading frame was cloned into the pMALcII expression vector (New England Biolabs, Inc).

Example 2 – Aerophilic and Aerophobic Responses

The resultant construct was transformed to *E. coli* pLysS cells for the expression and analyzed their behavior in a flat microcapillary using dark-field microscope coupled with time-lapse digital video system. Motile wild-type halobacterial cells form two clear congregated aerotactic bands, a positive one close to the interface between air and cell suspensions and a negative one away from the interface (Figure 5A, wild type). The positive aerotactic band is mediated by HtrVIII (Brooun et al., J. Bacteriol., 180:1642-1646 (1998), which is hereby incorporated by reference). As expected, this phenomenon is absent in the *htrVIII* deletion strain (Figure 5A, HemAT-*Hs*+ΔHtrVIII). However, like the wild type strain, the ΔHtrVIII strain also demonstrates the negative aerotactic band. If negative aerotaxis behavior is related to HemAT-*Hs*, one would postulate that in the *hemAT-Hs* deletion strain, the negative aerotactic band would not form. Indeed, in the Δh*emAT-Hs* strain, in which the positive aerotactic band is present due to the receptor HtrVIII, the sharp boundary of the negative aerotactic band is absent (Figure 5A, Δh*emAT-Hs*). Furthermore, when HemAT-*Hs* is overexpressed (using a multicopy plasmid) in a ΔhtrVIII strain, halobacterial cells form a more pronounced negative aerotaxis boundary (Figure 5A, HemAT-*Hs*+ΔHtrVIII). These cells were repelled from the air/liquid interface much faster and created a denser aerotactic band than the aerotactically wild type or ΔHtrVIII strains containing genomic copy of *hemAT-Hs* (Figure 5A, wild type and HemAT-*Hs*+ΔHtrVIII). The aerophilic response in *B. subtilis* proceeds more rapidly than it does in *H. salinarum* (30 versus 180 min) because *B. subtilis* swims faster than *H. salinarum.* In the wild-type, an aerotactic band formed at the air interface (Fix. 5B). This band did not form in a strain from which all ten putative MCP-like transducers (Δten) were deleted (Fig. 5B). A strain lacking only HemAT-*Bs* showed an aerophobic response, indicating the presence of a second, unidentified aerotaxis receptor. To demonstrate the physiological function of HemAT-*Bs*

unequivocally, *hemAT-Bs* was overexpressed in a strain from which all *B. subtilis* transducer genes were deleted (Δten strain). When HemAT-*Bs* was overexpressed in the Δten strain, the aerophilic response was observed (Fig. 5b). These assays demonstrate that HemAT-*Bs* is involved in an aerophilic response in *B. subtilis*.

Example 3 – Expression of HemAT-*Hs* and HemAT-*Bs* in *Escherichia coli*

The FAD-binding aerotaxis transducer Aer in *E. coli* has a PAS domain that is similar to the redox-sensing domain of the NifL protein of *Azotobacter vinelandii* (Hill et al., Proc. NatL Acad Sci. USA, 93:2143-2148 (1996); Zhulin et al., Mol. Microbiol., 29:1522-1523 (1998), which are hereby incorporated by reference) and FixL from *R. meliloti* (Gilles-Gonzalez et al., Nature, 350:170-172 (1991), which is hereby incorporated by reference). FixL is a chimeric membrane protein with a histidine kinase domain, which belongs to the large class of two-component regulatory systems, whereas the heme-binding sensory domain belongs to the PAS domain super family (Gilles-Gonzalez et al., Nature, 350:170-172 (1991); Lois et al., J. Bacteriol., 175:1103-1109 (1993); Gong et al., Proc. Natl. Acad. Sci. USA, 95:15177-15182 (1998), which are hereby incorporated by reference). None of the PAS domains identified in the genome of *B. subtilis is* present in chemotaxis transducers (Zhulin et al., Mol. Microbiol., 29:1522-23 (1998), which is hereby incorporated by reference). To identify the nature of the prosthetic groups in HemAT-*Hs* and HemAT-*Bs*, both proteins were expressed in *E. coli* by constructing vectors, which express the *hemAT-Hs* or *hemAT-Bs* gene under the control of an inducible T7 promoter (Studier et al., Methods in Enzymology, 185:60-89 (1990), which is hereby incorporated by reference).

Using a combination of anion exchange and gel-filtration chromatography, HemAT-*Hs* was purified (The BL21 pLysS host cells harboring *hemAT-Hs* or *hemAT-Bs* genes were grown to OD_{600} = 0.4 in 1L of LB with appropriate antibiotics and induced with 0.6 mM IPTG. The cells were harvested by low speed centrifugation (4000xg) for 15 min. at 4°C after a two-hour induction. The pellets were resuspended in 50 ml buffer (50 mM NaCl, 50 mM Tris-HCl, pH6.0) and sonicated for a total of 4 minutes (20 second pulses with 30 second pauses). The sonicated solution was centrifuged at 28,000xg for 20 min. The brown red supernatant with HemAT-*Hs* or HemAT-*Bs* was used for purification. **HemAT-*Hs*:** The supernatant was filtered through 0.2 micron filter and

applied to BioCAD anion exchange POROS HQ/M (16/100) perfusion chromatography column equilibrated with 50 mM Tris-HCI, pH6.0. A linear gradient of NaCl (0-1500 mM) was applied and HemAT-*Hs* was eluted at about 400 mM. For further purification, the fractions containing the HemAT-*Hs* (monitored by Soret band absorbence at 410 nm and SDS-gel electrophoresis) were concentrated and applied to a Hiload Superdex 200 16/60 gel filtration column. Peak fractions were concentrated with an Amicon l00K concentrator and used for spectroscopy. **HemAT-*Bs*:** A saturated $(NH_4)_2SO_4$ solution was added to the brown red supernatant to 30% and centrifuged at 28,000xg for 20 min. The optically clear light brown supernatant was further fractionated by $(NH_4)_2SO_4$ addition to 36% saturation followed by centrifugation. The resultant pellet was solubilized in a resuspension buffer (500mM NaCl, 50mM Tris-HCI, pH8) and applied to a Hiload 26/60 Superdex 75 gel filtration column. Peak fractions containing HemAT-*Bs* (monitored by Soret band absorbence at 410 nm and SDS-gel electrophoresis) were concentrated by an Amicon 50K concentrator and used for spectroscopy). Recombinant HemAT-*Hs* expressed in *E. coli* under low ionic strength conditions was shown to contain a high degree of secondary structure consistent with a predicted folded protein (Larsen et al., J. Prot. Chem., 18(3) (1999), which is hereby incorporated by reference).

The purified HemAT-*Hs* migrates at a position higher than the calculated 52.8 kDa for the mature protein (Figure 2B line HemAT-*Hs*). This slow electrophoretic migration in SDS-polyacrylamide gels is consistent with the highly acidic nature of HemAT-*Hs* (pI=3.78, 27% acidic residues) and has been observed in other acidic proteins from halophiles (Ihara et al., Arch. Biochem. Biophys., 286:111-116 (1991), which is hereby incorporated by reference). Using a combination of ammonium sulfate precipitation/fractionation and gel filtration chromatography it is possible to purify HemAT-*Bs*. The BL21 pLysS host cells harboring *hemAT-Hs* or *hemAT-Bs* genes were grown to OD_{600} = 0.4 in 1L of LB with appropriate antibiotics and induced with 0.6 mM IPTG. The cells were harvested by low speed centrifugation (4000xg) for 15 min. at 4°C after a two-hour induction. The pellets were resuspended in 50 ml buffer (50 mM NaCl, 50 mM Tris-HCl, pH6.0) and sonicated for a total of 4 minutes (20 second pulses with 30 second pauses). The sonicated solution was centrifuged at 28,000xg for 20 min. The brown red supernatant with HemAT-*Hs* or HemAT-*Bs* was used for purification. **HemAT-*Hs*:** The supernatant was filtered through 0.2 micron filter and applied to BioCAD anion exchange POROS HQ/M (16/100) perfusion chromatography column

equilibrated with 50 mM Tris-HCI, pH6.0. A linear gradient of NaCl (0-1500 mM) was applied and HemAT-*Hs* was eluted at about 400 mM. For further purification, the fractions containing the HemAT-*Hs* (monitored by Soret band absorbence at 410 nm and SDS-gel electrophoresis) were concentrated and applied to a Hiload Superdex 200 16/60 gel filtration column. Peak fractions were concentrated with an Amicon 100K concentrator and used for spectroscopy. **HemAT-*Bs*:** A saturated $(NH_4)_2SO_4$ solution was added to the brown red supernatant to 30% and centrifuged at 28,000xg for 20 min. The optically clear light brown supernatant was further fractionated by $(NH_4)_2SO_4$ addition to 36% saturation followed by centrifugation. The resultant pellet was solubilized in a resuspension buffer (500mM NaCl, 50mM Tris-HCI, pH8) and applied to a Hiload 26/60 Superdex 75 gel filtration column. Peak fractions containing HemAT-*Bs* (monitored by Soret band absorbence at 410 nm and SDS-gel electrophoresis) were concentrated by an Amicon 50K concentrator and used for spectroscopy). The purified HemAT-*Bs* migrates in SDS-PAGE as 48.7 kDa protein as expected (Figure 2, line HemAT-*Bs*).

Example 4 – Absorption Spectra of Purified HemAT-*Hs* and HemAT-*Bs*

HemAT-*Hs* and HemAT-*Bs* display similar absorption spectra in both the near UV and visible regions characteristic of oxygen bound heme proteins. Specifically, absorption band maxima are found at 406 nm (Soret), 578 nm (α-band), and 538 nm (β-band) for both proteins (Figure 4A). These absorption maxima resemble those of Sperm whale oxymyoglobin (418 nm, 581 nm, and 543 nm) and oxy FixL (415 nm, 577 nm, and 543 nm). Upon deoxygenation (using sodium dithionite), the Soret bands shift to 425 nm while the α- and β-bands converge to a broad band centered at 555 nm, consistent with the formation of a deoxy-form of the protein (i.e., absorption bands for deoxymyoglobin: 434 nm and 556 nm and deoxyFixL: 433 nm and 567 nm) (Figure 4B). If the deoxy form of HemAT-*Hs* and HemAT-*Bs* are exposed to atmospheric oxygen, the absorption spectra revert back to that observed for the purified proteins (Figure 4D). Both the purified (oxy form) and the deoxy derivatives of HemAT-*Hs* and HemAT-*Bs* are reactive towards carbon monoxide. The CO bound derivatives display absorption maxima at 415 nm (Soret), 573 nm (α-band), and 535 nm (β-band) (Figure 4C). A pyridine hemochrome

assay showed the heme group of both HemAT-*Hs* and HemAT-*Bs* to be b-type. HemAT-*Hs* and HemAT-*Bs* are distinct both in spectral features and in physiological function from the previously discovered heme protein FixL from *R. meliloti* (Gilles-Gonzalez et al., Nature, 350:170-172 (1991), which is hereby incorporated by reference). The absorption bands of both HemAT-*Hs* and HemAT-*Bs* are blue shifted, relative to FixL, indicating distinct heme pocket geometries. Unlike FixL, HemAT-*Hs* and HemAT-*Bs* display no PAS domain sequence homology. In addition, both HemAT-*Hs* and HemAT-*Bs* participate in negative aerotaxis while FixL acts as an oxygen sensing kinase.

Example 5 - Methylation of HemAT-*Hs* and HemAT-*Bs*

It has been postulated that in *E. coli*, adaptation in Aer-mediated aerotaxis is methylation-independent (Taylor et al., Annu. Rev. Microbiol., 53:90-103 (1999), which is hereby incorporated by reference). In contrast to *E. coli,* adaptation during aerotaxis in *H. salinarum* and *B. subtilis* is a methylation-dependent process (Brooun et al., J. Bacteriol., 180:1642-1646 (1998); Lindbeck et al., Microbiology, 141:2945-2953 (1995); Wong et al., J. Bacteriol., 177:3985-3991 (1995), which are hereby incorporated by reference). To determine if HemATs can be methylated by the CheR methyltransferase, *H. salinarum* and *B. subtilis* cells were radiolabeled with [*methyl-*^{3}*H*] methionine after blocking protein synthesis. The radiolabeled cells were processed for fluorography and immunoblotting with a polyclonal antibody raised against the highly conserved region of methyl-accepting transducers (W. Zhang et al., Proc. Natl. Acad. Sci. U.S.A. 93:4649 (1996), which is hereby incorporated by reference). A single radiolabeled band is missing in the Δ*hemAT-Hs* strain (Figure 2B, lane 1), whereas this band is present in the overexpression strain (Figure 2B, lane 2). This band is also recognized by the antibody, suggesting that HemAT-*Hs* is indeed a methyl-accepting transducer (Figure 2B, lanes 1' and 2'). In contrast, it was not possible to detect any [*methyl-*^{3}*H*]-labeling in HemAT-*Bs*. Together with the capillary assays, these data demonstrate an important difference in the signaling and adaptation mechanisms for aerotaxis mediated by HemAT-*Hs* and HemAT-*Bs*.

Example 6 – Materials and Methods for Example 7

PCR and TOPO cloning. C-terminal primers were designed to amplify the 250,230,210, 205, 200, 195, 194, 193, 192, 191, 190, 170 and 151 residues of HemAT-*Hs* and were designed to include *BamH*I and *Xba*I restriction sites. The N-terminal primer included *EcoR*I and *Nde*I restriction sites. Primer sequences may be found in Table 1. HemAT-*Hs* genomic plasmid was used as a template for PCR with *Pfu* polymerase. PCR amplification was performed in a GeneAmp PCR system 2400 (Perkin-Elmer) under the following conditions: Hot start with *Pfu* polymerase at 80°C followed by heat denaturation at 94°C for 2 minutes was followed by 25 cycles of heat denaturation at 94°C for 30 seconds, primer annealing at 58°C for 30 seconds and elongation at 72°C for 40 seconds. Following the last cycle, samples were maintained at 72°C for 7 minutes and immediately kept at 4°C. Following PCR, the PCR product was immediately cloned into the TOPO vector (Invitrogen) and transformed into TOP10 competent cells. Clones with the insertion were selected via kanamycin resistance on Luria Bertani (LB) agar plates with kanamycin (50μg/ml). Colonies were inoculated into CircleGrow (BIO101) with kanamycin media and, following incubation, plasmids were isolated via alkaline mini prep. Plasmids were then restricted with *EcoR*I to screen for the proper insert.

Cloning into pMAL expression vector. Plasmids containing the correct insert and the expression vector, pMAL-c2, were then digested with *EcoR*I and *BamH*I. pMAL-c2 was subsequently dephosphorylated with Alkaline Phosphatase. Digested TOPO plasmids and pMAL plasmid were run on a 1% preparative agarose gel. The truncated *hemAT-Hs* PCR insert and double digested pMAL-c2 bands were cut from the gel and the DNA was extracted from the gel using the GENECLEAN Spin Kit (BIO101). The *hemAT-Hs* insert was then ligated to the pMAL-c2 vector at 14°C, overnight. Following ligation, the ligation mixture was transformed into JM109 competent cells. Clones containing the plasmid were selected for by ampicillin resistance on LB agar Amp (100μg/ml) plates. Ampicillin resistant colonies were inoculated into CircleGrow + Amp media and incubated. Plasmids were isolated via alkaline mini prep and the *hemAT-Hs* insert was screened for by double digest with *EcoR*I and *BamH*I.

Transformation into expression host and protein expression. Plasmids containing the insertion were then transformed into BL21 p*LysS* competent cells

(Novagen). Clones containing both the pMAL-*hemAT-Hs* insertion plasmid and the pLysS plasmid were screened for by ampicillin (100μg/ml) and chloramphenicol (34μg/ml) resistance on LB agar plates. To check for expression of the truncated MBP-HemAT-*Hs* fusion protein, cells were inoculated into LB Amp and Chl broth and grown to an OD_{600}=0.4 followed by induction with 1 mg/ml IPTG. After induction for 1.5 hours, protein samples of uninduced and induced cultures were prepared and run on a 10% SDS-PAGE. This was then followed by staining for protein with Coomasie Blue and destaining with 10% acetic acid.

Protein purification by affinity chromatography and spectral analysis. Cultures which showed induction of the MBP-HemAT-*Hs* protein were then grown up in a larger scale to OD_{600}=0.4 and induced with IPTG (1 mg/ml). Induced cultures were then centrifuged at 5,000 rpm for 20 minutes at 4°C followed by a wash with column buffer (20mM Tris-HCI, 200mM NaCl, 1mM EDTA) and centrifuged again at 5,000 rpm for 20 minutes at 4°C. If purification did not immediately follow the wash, protein pellets were stored at -70°C. Protein pellets were then resuspended in column buffer, sonicated for 2 minutes (20 second pulses at 45 second intervals) resuspended in column buffer, sonicated for 2 minutes (20 second pulses at 45 second intervals) and centrifuged at 15,000 rpm for 20 minutes at 4°C. The protein containing supernatant was decanted, diluted 1:2 and stored on ice. After setting up the amylose resin column (New England BioLabs), it was washed with 8 column volumes of cold column buffer. The sample was then loaded onto the column at a flow rate of 1 ml/min. followed by a 12 column volume wash with cold column buffer. MBP-HemAT-*Hs* protein was eluted with 10 mM maltose column buffer and collected in 1 ml fractions. Samples containing the most protein were used to determine the spectra via spectrophotometer. Following elution, a 10% SDS-PAGE was also often run to determine the amount of protein in elutions. Eluted samples were stored at 4°C.

Table 3. Names and sequences (5' to 3') of primers used in HemAT-*Hs* truncation.

Primer Name	Sequence (5' to 3')	
hemAT-Hs EcoRI/NdeI top	ccgaattccatatgagcaacgataatgac	SEQ. ID. No. 48
hemAT-Hs 151 *BamHI/XbaI* bot	ctctagaggatccctagtcgtcggcaagcgcgtcc	SEQ. ID. No. 49
hemAT-Hs 250 B/X bot	cctctagaggatccctagacgtcagccatgcggtc	SEQ. ID. No. 50
hemAT-Hs 230 B/X bot	cctctagaggatccctaggcgacgtcctgcgaggtcgcc	SEQ. ID. No. 51
hemAT-Hs 210 B/X bot	cctctagaggatccctacgcgttcgccaactcctggcggc	SEQ. ID. No. 52
hemAT-Hs 190 B/X bot	cctctagaggatccctagatgtaggtgtccattgcgatc	SEQ. ID. No. 53
hemAT-Hs 170 B/X bot	cctctagaggatccctaccgggccacgagttcgtcgac	SEQ. ID. No. 54
hemAT-Hs 205 B/X bot	cctctagaggatccctactggcggctgtcgatctcgtc	SEQ. ID. No. 55
hemAT-Hs 200 B/X bot	cctctagaggatccctactcgtcgtggaggcgctgggc	SEQ. ID. No. 56
hemAT-Hs 195 B/X bot	cctctagaggatccctactgggcgtacgagtcgatgtag	SEQ. ID. No. 57
hemAT-Hs 194 B/X bot	cctctagaggatccctaggcgtacgagtcgatgtaggtgtcc	SEQ. ID. No. 58
hemAT-Hs 193 B/X bot	cctctagaggatccctagtacgagtcgatgtaggtgtcc	SEQ. ID. No. 59
hemAT-Hs 192 B/X bot	cctctagaggatccctacgagtcgatgtaggtgtccattgcg	SEQ. ID. No. 60
hemAT-Hs 191 B/X bot	cctctagaggatccctagtcgatgtaggtgtccattgcg	SEQ. ID. No. 61

Example 7 – Truncated HemAT-*Hs*

The finding that HemAT-*Hs*, an archael signal transducer, is a heme binding protein provides a unique opportunity to study not only the physiological function of this protein, but also obtain greater understanding of the structure of this soluble protein and how heme interacts with it. Therefore, this project aims to identify the minimum size of HemAT-*Hs* to which heme binds. This will be done by truncating the gene, first from the C - terminal, by PCR. Once the minimum size of the functional heme binding domain is found from the C - terminal, the N - terminal will then be truncated to further identify residues crucial in proper heme binding. Producing this truncated HemAT-*Hs* protein which still retains the functional heme binding domain will aid in efforts to determine HemAT-*Hs* protein structure.

Analysis of heme binding in HemAT-*Hs* began with the first 151 residues of HemAT-*Hs*. However, preliminary spectral analysis showed no heme binding. Primers were then designed to amplify HemAT-*Hs* every 20 amino acids from 150 thereby amplifying the first 170, 190, 210, 230, and 250 amino acids of the N-terminal. HemAT-*Hs* 210 showed the spectra of heme bound to HemAT-*Hs* and also exhibited the characteristic red spectra of O_2 bound heme in purified protein samples. HemAT-*Hs* 190, however, did not present color in protein samples, nor did it have the visible bands at 540 nm and 580 nm which represent bound heme. Primers were then designed every 5 amino

acids from 210 to 190 at 205, 200 and 195 to determine more precisely where heme binds. Only 200 and 195 construction fused with MBP showed a reddish color in protein samples along with the characteristic spectra.

This narrowed the search down for the heme binding site to between 195 and 190 amino acids of HemAT-*Hs*; thus, primers were designed at 191, 192, 193 and 194 amino acids. The spectra for the 192 and 191 constructs shows altered visible bands at 540nm and 580nm. The 194 construction shows a similar spectra, like wild-type HemAT-*HS* or the HemAT-*Hs* 195 construct.

Example 8 – Purification of Recombinant HemAT-*Hs* by Metal Chelate Chromatography

1 L of *E. coli* culture containing HemAT-*Hs* was collected, washed with Buffer #2 (200 MM NaCl, 50 mM Sodium phosphate, pH 8.0), and resuspended in 40 ml of Buffer #2. Cells are sonicated. Insoluble material is removed by ultracentrifugation at 100,000 rpm for 20 minutes. POROS MC/M (100 X 1.6 I.D., 20 μm) is used for metal chelate chromatography. The column is washed with 50 mM EDTA, I M NaCl, pH 8.0 over 10 column volumes followed by a wash with water. 100 mM $CoCl_2$ is used to charge the column, followed with a wash with 1 M NaCl and water. The column is equilibrated in buffer containing 5 mM imidazole. 5 ml of sample is loaded directly onto the column at a flow rate of 2-4 ml/min and a gradient of imidazole from 0-500 mM is run over 30 column volumes at 10 ml/min. Fractions containing recombinant HemAT-*Hs* is pooled and concentrated using Centricon 50.

Example 9 - Site Directed Mutagenesis of HemAT-*Hs*

In order to perform PCR-based site-directed mutagenesis, a plasmid containing the *hemAT-Hs* gene to be mutated with proper size has to be constructed first. The proper restriction recognition sites are created by designing the primers with the recognition site tags in the primers as follows:

*hemAT-Hs EcoRI/Nde*I top primer:

5' CCGAATTCCATATGAGCAACGATAATGAC 3' (SEQ. ID. No. 62)

*hemAT-Hs BamHI/Xba*I bottom primer:

5' CCTCTAGACTAGCTGAGCTTGCCGACC 3' (SEQ. ID. No. 63)

Two sites in each primer were created to meet the needs of expressing HemAT-*Hs* in different expression vectors. *hemAT-Hs* genomic DNA in pDelta vector was used as a template for amplifying *hemAT-Hs* gene by PCR using proofreading DNA polymerase *pfu*. PCR product was cloned into TOPO vector (Invitrogen TOPO cloning Kit). The insert was checked and confirmed by digestion and PCR. This construction was used as template for generating serial histidine mutants.

The plasmid construction from above was used for mutagenesis PCR. His 20, His 71, His 123, His 198, and His 214 were mutated to alanine by PCR-based site-directed mutagenesis (described above). Mutated *hemAT-Hs* gene in Topo vector has been checked by manual sequence as well as Auto Sequencer 373.

Table 4: Primers for mutagenesis.

Primer Name	Sequence	
H20A	GGAACGGGATCGACGGGgccGCACTCGCGGACCGG	SEQ. ID. No. 64
H20A-R	CCGGTCCGCGAGTGCggcCCCGTCGATCCCGTTCC	SEQ. ID. No. 65
H70A	GACCGACTTCTACGACgccTTGGAGTCCTACGAGCG	SEQ. ID. No. 66
H70A-R	CGCTCGTAGGACTCCAAggcGTCGTAGAAGTCGGTC	SEQ. ID. No. 67
H123A	CCGTATCGGGAAGATAgccGACGTGCTCGGGCTCG	SEQ. ID. No. 68
H123A-R	CGAGCCCGAGCACGTCggcTATCTTCCCGATACGG	SEQ. ID. No. 69
H198A	CGTACGCCCAGCGCCTCgccGACGAGATCGACAGCC	SEQ. ID. No. 70
H198A-R	GGCTGTCGATCTCGTCggcGAGGCGCTGGGCGTACG	SEQ. ID. No. 71
H214A	GCGAACGCGGTCGCCACGgccGTGGAAGCACCGCTG	SEQ. ID. No. 72
H214A-R	CAGCGGTGCTTCCACggcCGTCYGCGACCGCGTTCGC	SEQ. ID. No. 73

Total of **10** mutants have been done, including H20A, H70A, H123A, H198A, H214A, H20/70A, H20/123A, H70/123A, H20/70/123A.

Example 10 - Expression of mutated *hemAT-Hs*

The *hemAT-Hs*/pTOPO construction was used as initial plasmid for the subcloning of *hemAT-Hs* gene into different vectors. Three different expression systems were used. First, the pMAL system was used for expression in *E. coli* (Fusion protein expression system). *EcoR*I and *BamH*I restriction digestion sites were used for cloning the mutated *hemAT-Hs* gene into pMAL vector. The protein expressed in this system is a MBP HemAT-*Hs* fusion protein. All of the mutants have been cloned into pMAL, expressed successfully, purified and spectra have been done as well.

Second, the pET system is also used for expression of the peptides in *E. coli*. *Nde*I and *BamH*I restriction digestion sites were used for subcloning *hemAT-Hs* into pET vector.

Third, in order to study the physiological function of HemAT-*Hs* in its native host, it has to be expressed in halobacterial Δ*hemAT-Hs* strain, a strain that *hemAT-Hs* gene has been deleted from its genome. *Nde*I and *Xba*I were used to clone mutated *hemAT-Hs* gene into a halobacterial shuttle expression vector pKJ427. *hemAT-Hs*/pTOPO plasmid was digested with *Nde*I and *Xba*I, as well as the shuttle vector pKJ427. Digested vector and *hemAT-Hs* insert were purified from agarose gel by GeneClean kit and ligated with T4 ligase at 4°C. Ligation reaction was transformed into *E. coli* competent cells. Colonies were inoculated, the plasmids were extracted and checked by double digestion and PCR. The final construction was transformed into halobacterial *hemAT-Hs* deletion strain for over-expressing HemAT-*Hs* in *H. salinarum* (standard halobacterial transformation protocol was used). Cultures were checked for expression of HemAT-*Hs* by immunoblot using both HC23 antibody and HemAT-*Hs* specific antibody. The clone with highest expression of HemAT-*Hs* was used for physiological study.

Example 11 – Construction of a C-terminal His-tag of *hemAT-Hs*

In order to purify HemAT-*Hs* protein from its native host *Halobacterium salinarum* C-terminal His-tag was constructed. A two-step PCR strategy was used. First, an *Nde*I top primer and 20 nucleotide C-terminal of *hemAT-Hs* gene plus sequence

encoding 6-histidine primer were used for amplification. Second, using first round PCR product as template, *Nde*I top primer and 6-Histidine + Stop codon bottom primer were used for PCR. The primer (including *BamHI*/*Xba*I cutting sites) was used to amplify the *hemAT-Hs* gene plus histidine codon as well as stop Codon right after 6-his sequence. TOPO cloning was used for cloning the PCR products. *Nde*I/*Xba*I were used for subcloning of *hemAT-Hs*-6-His-stop construction into shuttle vector pKJ427. The final construction plasmid was transformed into *hemAT-Hs* deletion strain.

Example 12 - HemAT-*Hs* overexpression construction in *H. salinarum*

Expression of HemAT-*Hs* in *Halobacterium salinarum* was created in the expression vector pKJ 427. pKJ 427 plasmid contains a fedox promotor with an mevinolin resistant gene. *Nde*I and *Xba*I restriction recognition sites were used to clone the *hemAT-Hs* gene into the pKJ427 vector. Top primer with *Nde*I cutting site and bottom primer with *Xba*I cutting site were designed and used for amplifying *hemAT-Hs* gene from *Halobacterium salinarum* genomic DNA by proof-reading pfu DNA polymerase. The PCR product was cloned into TOPO cloning vector (Invitrogen) and transformed into *E. coli* competent cells. The plasmid containing *hemAT-Hs* gene in TOPO vector was subcloned into pKJ 427 vector by *Nde*I/*Xba*I double digestion. The *hemAT-Hs*/pKJ427 construction was confirmed by PCR as well as *Nde*I/*Xba*I double digestion and then, the plasmid was transformed into Δ*htrVIII* deletion strain using standard transformation protocol. After two week incubation, colonies were picked up and grown in halobacterial growth medium. Each individual culture was checked by PCR to confirm the presence of the plasmid, and by immunoblot to confirm the expression level of HemAT-*Hs*.

Example 13 – Expression of *hemAT-Bs*

As with *hemAT-Hs*, three expression systems have been developed. First, *hemAT-Bs* is expressed in the pMAL expression system. In order to express *hemAT-Bs* encoding protein HemAT-*Bs* in *E. coli*, expression primers were needed to amplify the *hemAT-Bs* gene. Not only the gene, but the ribosomal binding region upstream of the start codon, is required for the expression of HemAT-*Bs* in *E. coli*.

Table 5: Primers for PCR of HemAT-*BS*.

Name of Primer	Sequence
hemAT-Bs *BamH*Itop	ATATGGATCCAAGGGGGATCATTGTAATGTTATTTAAAAAAG SEQ. ID. No. 74
hemAT-Bs *Pst*Ibot	ATTACTGCAGCAACTGATTTTTAATTTAAGTTTACATAATGAACGC SEQ. ID. No. 75

*BamH*I and *Pst*I were selected for the cloning of *hemAT-Bs* into expression vector and *E. coli-Bacillus subtilis* shuttle vector. *hemAT-BsBamH*I *top/Pst*I bot primers were used to amplify the *hemAT-Bs* gene from *Bacilus subtilis* gemonic DNA by PCR with *pfu* DNA polymerase. After PCR, the amplicon was immediately cloned into TOPO vector using invitrogen TOPO Blunt Cloning kit and transformed into TOP 10 *E. coli* competent cells. Colonies were checked for the right insert.

*BamH*I and *Pst*I were also used for the cloning of the HemAT-*Bs* into pMAL cII vector as well as the shuttle vector pEB 112. *hemAT-Bs*/pMAL construction was transformed to *E. coli* pLysS cells for the expression. After IPTG induction, SDS gel showed two bands in comparison to the uninduced sample. The top band is HemAT-*Bs* protein. The spectra is checked and the results showed clearly the hemeprotein signature peaks while the MBP itself doesn't show any peak at 410 nm and 541nm/580nm.)

Second, the *Nde*I top and *BamH*I bot primers were used for the cloning of *hemAT-Bs* gene into pET vector. The ribosomal binding region is also included in front of the gene. TOPO cloning was performed after PCR reaction and the construction was confirmed by *NdeI/BamH*I digestion as well as PCR. *hemAT-Bs*/pET construction was transformed into *E. coli* pLysS competent cells. IPTG was used for the protein induction. Spectra showed the specific peaks for hemeprotein.

A peptide consisting of the N-terminal 190 or 250 residues was expressed in pMAL vector. A bottom primer at position 190 and 250 amino acid residue were synthesized with a *Pst*I cutting site. *hemAT-Bs BamH*I top and these top primers were used to amplify the gene encoding 190 and 250 amino acids at N-terminal of *hemAT-Bs*. The PCR products were cloned into TOPO vector and then subcloned into pMAL HemAT-*Bs* 250 vector by using *BamHI/Pst*I. 190 and 250 *hemAT-Bs*/pMAL constructions were confirmed and transformed into pLysS cells for expression. As

expected, other than MBP, a second protein band appears at position 25 and 30 kDa, which are the sizes of N-terminal 190 and 250 residues of HemAT-*Bs* protein. Spectra also showed the signature peaks of hemeprotein.

A shuttle vector is used for the expression of *hemAT-Bs* gene in its native host *B.subtilis*. *hemAT-Bs*/TOPO construction was used as initial plasmid. The *hemAT-Bs*/pEB 112 construction was transformed into Δ*hemAT-Bs* deletion strain. The transformant was used for physiological study of HemAT-*Bs*.

Example 14 - Construction of a C-terminal His-tag HemAT-*Bs*

Two round of *pfu* PCR were performed to generate a C-terminal 6 His-tag to HemAT-*Bs*. The top primer and bottom primer with 6 Histidine codon plus stop codon were used for the first round PCR. The PCR product was cloned into TOPO vector and the resultant vector used for the second round PCR. In the case of pET, *Nde*I and *BamH*I sites were created to clone the insert into expression vector. In the case of pMAL, *BamH*I top and *BamH*I bot primer were used. The final constructions (pET/pMAL) were transformed to *E. coli* pLysS cells for induction.

Example 15 - Site-Directed Mutagenesis of HemAT-*Bs*

The same strategy is used for generating site-directed mutants for HemAT-*Bs* of *B. subtilis*. The HemAT-*Bs*/TOPO construction with *BamH*I top and *Pst*I bottom restriction sites was used as template for PCR-based mutagenesis. HemAT-*Bs* H75A, H86A, H99A, H122A, H123A and H199A are being mutated by PCR-based mutagenesis. The HemAT-*Bs*/pTOPO plasmid was used as initial template for PCR.

The mutants, H75A, H99A, and H123A, have been cloned into pMAL expression vector. H123A spectra showed no significant signature peaks at 540 nm and 580 nm. H123R from pMAL expression culture showed no hemeprotein signature spectra.

Example 16 –Carbon Monoxide Binding in HemAT-*Hs* and HemAT-*Bs*

The rate of CO binding to both HemAT-*Hs* and HemAT-*Bs* was determined by transient absorption spectroscopy using instrumentation described previously (Larsen,et al., Inorg. Chim. Acta 234:101-107(1995), which is hereby incorporated by reference).

The rates of CO dissociation for HemAT-*Hs* and HemAT-*Bs* were determined using the ferricyanide method (Gilles-Gonzalez, et al., Biochemistry 33:8067-8073 (1994), which is hereby incorporated by reference). Changes in absorbance as a function of time at 418 nm (Soret maximum for the CO bound derivative of each protein) were monitored after the addition of potassium ferricyanide (final concentration of 1.5 mM) to solutions of the co-bound protein. The resulting traces were then fit to single exponential decays to obtain k_{off} assuming the following reaction:

$$\text{CO-Fe(II)HemAT-Xx} \underset{K_{on}}{\overset{K_{off}}{\longleftrightarrow}} \text{Fe(II)HemAT-Xx} + \text{CO} \xrightarrow{\text{Fe(III)(CN-)6,k1}} \text{Fe(III)HemATs}$$

where k_{off}/k_{on} are the dissociation/association rate constants and k1 is the rate of HemATs oxidation. This procedure relies on k1 being much larger than k_{off}. In the case of the HemATs proteins this was confirmed by measuring the rate of heme oxidation of the five-coordinate deoxy form the protein.

The optical absorption spectrum of deoxy and CO-bound derivatives of HemAT-*Hs* and HemAT-*Bs* are shown in Figure 4. The absorption spectra of the deoxy forms of both proteins are indicative of five-coordinate high-spin heme with Soret maxima at 425 nm and a broad visible band centered at 555 nm. In the presence of CO the absorption spectrum resembles a six-coordinate low-spin heme with a Soret maximum at ~418 nm (HemAT-*Hs/Bs*) and visible bands at 535 nm and 573 nm.

Figure 6 displays typical transient absorption data subsequent to CO photolysis obtained at 430 nm at 25°C and 1 atm CO for both HemAT-*Hs* (solid line) and HemAT-*Bs* (dotted line). The data can be fit to a single exponential decay indicating a pseudo-first order reaction with CO. The resulting rate constant for CO recombination are found to be 30±3 s^{-1} and 132±3 s^{-1} for HemAT-Hs and HemAT-*Bs*, respectively. Figure 7 shows

the corresponding transient difference spectrum (25 μs subsequent to photolysis) overlaid with the equilibrium difference spectrum (deoxy minus CO-bound) for HemAT-*Hs* (top panel) and HemAT-*Bs* (bottom panel). The red-shift in the transient difference spectra relative to the equilibrium difference spectra suggest that CO photolysis produces a non-equilibrium five-coordinate complex within 25 μs subsequent to photolysis.

Figure 8 displays the CO-off rate data for HemAT-*Hs*, HemAT-*Bs*, and horse heart Mb. The CO off-rates are found to be 0.2±0.01 s^{-1}, 0.098±0.002 s^{-1}, and 0.056±0.001 s^{-1} for HemAT-*Hs*, HemAT-*Bs*, and horse heart Mb, respectively. Using these values along with the second-order rate constants for CO recombination (scaling the pseudo first order rate constants to CO concentration) the associations constants for CO are found to be $1.5x10^4$ M^{-1}, $1.35x10^6$ M^{-1}, and $7.38x10^6$ M^{-1} for HemAT-*Hs*, HemAT-*Bs*, and horse heart Mb, respectively. These values along with literature values for CO binding to other heme proteins are provided in **Table 6**.

Table 6: CO-Affinities of various heme proteins.

Protein	K ($x10^{-4}$ M^{-1})	K_{on} ($x10^{-4}$ $M^{-1}s^{-1}$)	K_{off} (s^{-1})
HemAT-*Hs*[a]	15	3	0.2
HemAT-*Bs*[a]	135	13.2	0.098
HH Mb[a]	738	46.5	0.06
SW Mb[b]	2700	51	0.019
SW Mb H(E7)->L[b]	110,000	2,600	0.024
Human HbA[c]	50,000	600	0.013
BjFixL[c]	10	0.5	0.045
RmFixLT[c]	-	1.2	-
RmFixLH[c]	20	1.7	0.083
HRP (pH 7.0)[c]	350	0.3	0.0001
Aplaysia Mb[c]	3,000	50	0.02

[a] This work.
[b] Springer, et al., Chem. Rev. 94:699-714 (1994), which is hereby incorporated by reference.
[c] Gilles-Gonzalez, et al., Biochemistry 33:8067-8073 (1994), which is hereby incorporated by reference.

The absorption spectra of oxy-, deoxy-, and carbon monoxide forms of HemAT-*Hs* and HemAT-*Bs* establish that both proteins have a heme prosthetic group to reversibly bind oxygen. Capillary assays demonstrate that both HemAT-*Hs* and HemAT-*Bs* are

involved in negative aerotaxis in phylogenetically distinct archaeon *H. salinarum* and gram-positive bacterium *B. subtilis,* respectively. Thus, the N-terminal segments of HemAT-*Hs* and HemAT-*Bs* may act as sensory domains by binding diatomic oxygen through the heme prosthetic group in the ferrous (Fe(II)) state. This oxygen binding triggers a conformational change in the sensor domain, which in turn alters the activity of the C-terminal signaling domain. This initiates association of the signaling domain with CheW and CheA proteins to generate signals that change the flagellar rotational bias.

Current evolutionary reconstruction indicates that myoglobin, (α- and β-globins derive from a protein that originally appeared in an ancient vertebrate about 500 million years ago (Hardison, Amer. Scientist, 87:126-137 (1999), which is hereby incorporated by reference). However, comparison of amino acid sequences in globins from *Eukarya* and *Bacteria* suggests they share a very early common ancestor, in spite of the fact that the proteins perform different functions (Hardison, Amer. Scientist, 87:126-137 (1999); Hardison, J. Exp. Biol., 201:1099-1117 (1998), which are hereby incorporated by reference). The conserved residues among all myoglobins are the proximal histidine residue in the F helix (F8) and two phenylalanine residues in the CD region (CD1 packs against the heme and CD4 in a hydrophobic cluster in contact with the heme), the distal histidine residue in the E helix (E7) and a proline residue at the beginning of the C helix (C2, sharp turn between B and C helices) (Bashford et al., J. Mol. Biol., 196:199-216 (1987); Vinogradov et al., Comp. Biochem. Physiol., 106B:1-26 (1993), which are hereby incorporated by reference). Three of these residues (proline in C2, phenylalanine in CD4 and histidine in F8) are conserved and phenylalanine in CD1 is replaced by valine in HemAT-*Hs* and HemAT-*Bs* (marked with asterisks in Figure 1A).

HemAT proteins constitute a new class of sensors that differ significantly from the known heme-containing O_2 -sensor FixL (16, 17). FixL is a member of the large family of sensor kinases ubiquitous in bacterial two-component regulatory systems. Its heme-binding domain belongs to the PAS-domain superfamily (18, 19). HemATs contain no PAS domains (Taylor, et al., Ann. Rev. Microbiol., 53:90 (1999); Zhulin et al., Mol. Microbiol., 29:1522 (1998), which are hereby incorporated by reference) and differ from FixL both in spectral features and physiological function (Gilles-Gonzalez, et al., Nature, 350:170 (1991); Lois, et al., J. Bacteriol., 175:1103 (1993), which are hereby incorporated by reference). The absorption bands of HemATs are blue-shifted relative to FixL (415 nm Soret band), indicating that the proteins have distinct heme-pocket

geometries. In addition, both HemATs participate in aerotaxis, whereas FixL regulates transcription. HemATs also differ from the aerotaxis transducer Aer in *E. coli*, which has a FAD-binding PAS domain (Rebbapragada et al., Proc. Natl. Acad. Sci. U.S.A., 94:10541 (1997); Bibikov et al., J. Bacteriol., 179:4075 (1997), which are hereby incorporated by reference).

The amino-terminal domains of HemATs are proposed to act as sensors by binding diatomic oxygen at their heme when it is in the ferrous (Fe [II]) state. Oxygen binding presumably triggers a conformational change in the sensor domain that, in turn, alters the activity of the carboxyl-terminal signaling domain. The carboxyl-terminal domains of HemATs are very similar to the signaling domains of the MCP family of bacterial chemoreceptors, which associate with the cytoplasmic CheW and CheA proteins to mediate chemotaxis.

HemATs offer the possibility of being used as biological sensors to monitor physiologically important gases, such as O2 or CO, because: 1) they are soluble proteins like myoglobin, which has been widely studied at the molecular level; 2) they possess a signaling domain that resembles those of the molecularly well-characterized bacterial chemotaxis transducers; and 3) direct observation of the aerotactic response permits rapid analysis of various perturbations of the sensing and signaling system. In addition, these two proteins provide information about the evolutionary origins of globins in the *Eucarya*, *Archaea*, and *Bacteria*.

Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.